

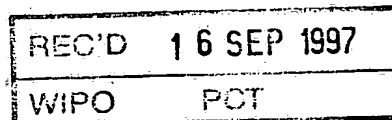


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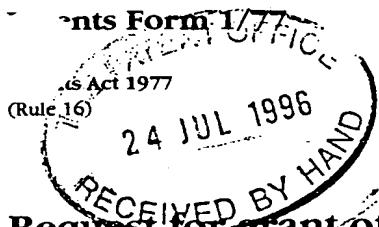
Signed

*Andrew Gersey*

Dated

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1/77  
24 JUL 96 E209761-7 001038  
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The Patent Office

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1. Your reference

24 JUL 1996

JPD/EE/UNIBR2

2. Patent application number

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9615551.0

3. Full name, address and postcode of the or of each applicant (underline all surnames)

The University of Bristol  
Senate House  
Tyndall Avenue  
Bristol BS8 1TH

Patents ADP number (if you know it)

798181002

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention

GALANIN

5. Name of your agent (if you have one)

Withers & Rogers

"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)

4 Dyer's Buildings  
Holborn  
LONDON EC1N 2JT

Patents ADP number (if you know it)

1776001

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and (if you know it) the or each application number

Country

Priority application number  
(if you know it)

Date of filing  
(day / month / year)

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application

Date of filing  
(day / month / year)

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? (Answer 'Yes' if:

- a) any applicant named in part 3 is not an inventor, or
  - b) there is an inventor who is not named as an applicant, or
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## Patents Form 1/77

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Continuation sheets of this form

Description 9

Claim(s) 3

Abstract 1 *NP*

Drawing(s) 9

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Priority documents

Translations of priority documents

Statement of inventorship and right to grant of a patent (*Patents Form 7/77*)

Request for preliminary examination and search (*Patents Form 9/77*) One

Request for substantive examination (*Patents Form 10/77*)

Any other documents  
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11. I/We request the grant of a patent on the basis of this application.

Signature

*Witthers & Rogers*  
WITHERS & ROGERS

Date 23/7/96

12. Name and daytime telephone number of person to contact in the United Kingdom

John Dean

0117-925 3030

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## GALANIN

This invention relates to galanin, including analogues thereof and its uses.

- 5 Galanin is a 29 amino acid neuropeptide which was first isolated from porcine intestine in 1983. Subsequently, the cDNA for galanin was cloned from a rat anterior pituitary library in 1987. Nucleotide and amino-acid sequence analysis suggests that galanin is unrelated to any of the other known families of regulatory peptides, and remains the only member of its family. The N-terminal portion of galanin is highly conserved between species, there being  
10 variation in the C-terminal portion

Galanin has a widespread distribution in the peripheral and central nervous systems, gut and pancreas. It is found in highest levels in the median eminence of hypothalamus and in the pituitary.

15

- WO92/12997 (General Hospital Corporation) published in 1992 discloses the sequence of human galanin. There is a discussion of studies by other workers involving the administration of rat galanin or its N-terminal fragments to augment the effect of morphine and this application suggests that galanin can be expected to exhibit analgesic effects such  
20 that it may be administered alone or in combination with other analgesics. The application claims the use of galanin or its analogues in the treatment of pain and the use of galanin antagonists in the treatment of other conditions.

- WO92/20709 (Astra AB) discloses a number of putative galanin antagonists. The  
25 antagonists which are described these are all based on the first 12 amino acids of galanin followed by partial sequences of other peptides i.e. chimeric peptides. Some may be agonists, some antagonists and some maybe both depending on the receptor subtype. The application discloses that the antagonists may be useful for treatment of insulin, growth hormone, acetyl choline, dopamine, Substance P, Somatostatin, Noradrenaline-related  
30 conditions including endocrinology, food intake, neurology and psychiatry, Alzheimer's type dementia, analgesia, intestinal disease. The application discloses the results of studies using some of the antagonists described therein on various effects such as galanin inhibition of glucose stimulated insulin release; galanin induced inhibition of scopolamine
- O:\SPJPD\Unibr2.lwp

induced ACh hippocampal release; galanin induced facilitation of the flexor reflex; the displacement of bound iodinated galanin in membrane binding studies. There is a suggestion in the application that the antagonists may be indicated for analgesia but there is no disclosure in the application of results to this effect.

5

Approximately 2-4% of the Western population suffer from diabetes mellitus and of those people 10-15% suffer from chronic pain and numbness in their extremities termed painful neuropathy. Present techniques for management of painful neuropathy are inadequate.

- 10 The present invention relates to the generation of a mouse with targeted disruption of the galanin gene; experiments using the mouse, and the implication of the results of those experiments for the treatment of disease.

According to one aspect of the invention there is provided a mammal, preferably a rodent,  
15 which lacks a functional galanin gene. The term galanin embraces all known galanins including, for example, human, rat, murine and porcine galanin and also analogues of galanin having the biological activity of galanin. The galanin gene may have been inactivated by at least partial deletion of the galanin gene sequence between the Bam HI and Bgl2 restriction sites indicated by asterisks in the accompanying Fig. 3. Where the  
20 mammal is a rodent it is preferably a mouse. Other mammals such as sheep and rats are contemplated.

According to another aspect of the invention there is provided tissue, cells and cell lines derived from the mammal in accordance with the first aspect of the invention. Preferably,  
25 the tissue, cells or cell lines include cells from pancreas, pituitary, cortex, dorsal root ganglia or are derived from such cells.

The mammal or tissue, cells and cell lines of the invention may be used in an assay to study a biological effect of galanin. The biological effect may be selected from, for  
30 example, prolactin secretion, appetite, memory, behaviour, pain, autotomy following axotomy, growth.

According to another aspect of the invention there is provided an analgesic composition comprising a galanin antagonist and in addition the use of a galanin antagonist in the preparation of a medicament for the treatment of pain.

- 5 According to a further aspect of the invention there is provided a method of suppressing pain in a mammal, the method comprising administering a galanin antagonist to that mammal and, in addition, the use of a galanin antagonist in the preparation of a medicament for the treatment of painful neuropathy.
- 10 According to a further aspect of the invention there is provided an appetite suppressant composition comprising a galanin antagonist and, in addition, the use of a galanin antagonist in the preparation of a medicament for the suppression of appetite. This aspect of the invention also provides a method of suppressing appetite in a mammal, the method comprising administering a galanin antagonist to that mammal.

15

According to a further aspect of the invention there is provided an anaesthetic composition comprising a galanin antagonist and, in addition, the use of a galanin antagonist in the preparation of an anaesthetic composition. This aspect of the invention also provides a method of anaesthetising a mammal, the method comprising administering a galanin antagonist to that mammal.

20

According to a further aspect of the invention there is provided the use of a galanin antagonist in the preparation of a medicament for the suppression of lactation and also a method of suppressing lactation in a mammal, the method comprising administering a galanin antagonist to that mammal.

25

According to a further aspect of the invention there is provided a composition comprising a galanin antagonist for the treatment of prolactinoma in a mammal and also the use of a galanin antagonist in the preparation of a medicament for the treatment of prolactinoma and a method of treating prolactinoma in a mammal suffering from prolactinoma, the method comprising administering a galanin antagonist to that mammal.

30

The generation of mutant mice in accordance with the invention will now be described, by way of example only, with reference to the accompanying drawings Figures 1 to 9 in which:

5 Fig. 1 illustrates the genomic structure of mouse galanin;

Fig. 2 illustrates the targeting vector used in producing the rodent of the invention;

Fig. 3 illustrates the specific recombination event in the production of the rodent in  
10 accordance with the invention;

Fig. 4 illustrates the effect of galanin disruption on the heat sensitivity of the rodent in accordance with the invention;

15 Fig. 5 illustrates the results of experiments on autotomy behaviour after sciatic nerve section;

Fig. 6 illustrates the effect of galanin inactivation on anterior pituitary prolactin content.;

20 Fig. 7 illustrates the effect of galanin inactivation on anterior pituitary thyroid stimulating hormone content;

Fig. 8 illustrates the effect of galanin inactivation on anterior pituitary growth hormone content; and

25

Fig. 9 illustrates the effect of galanin inactivation on anterior pituitary luteinizing hormone content .

30 To generate a mouse knockout, that is the introduction into the mouse genome of either a loss- or gain-of-function mutation of a specific gene locus ( according to the procedure described in Kuehn, M. R. *et al* Nature. 1987; 326: 295-8; Thomas, K. R. and Capecchi, M. R. Nature. 1986; 324: 34-8) , entails a number of steps:- (1) the cloning of the mouse



genomic locus of interest; (2) the construction of a targeting vector such that the locus/gene of interest is modified to inactivate or alter its structure and function in some way; (3) introduction of the targeting vector into an embryonic stem cell library and selection and identification of single cell clones in whom the appropriate correct targeting event has taken place and in whom the normal chromosomal number is unchanged; and (4) introduction of such clones into 3.5 day old blastocysts and the resulting chimeric mice mated to wild types of the opposite sex. The resulting offspring demonstrated to carry the mutation are thus heterozygotes and, by appropriate mating, homozygotes for the introduced mutation are bred.

10

As a first step the murine *galanin* gene was cloned. A mouse genomic library (Ehrich, E. *et al* Gene. 1987; 57: 229-37) was screened using the full length rat *galanin* cDNA as a probe under high stringency. Two cosmid clones were identified spanning 60Kb around the *galanin* locus. Using 5' and 3' probes from the rat cDNA a 14 Kb region of DNA containing the entire gene was subcloned and partially sequenced. From the genomic sequence, primers were designed complementary to untranslated exonic regions of the gene. A 630bp fragment was generated by RT-PCR (Kit supplied by INVITROGEN BV, The Netherlands) using adult female whole brain as a source of mRNA. Subsequent sequencing of this fragment demonstrated that mouse and rat *galanin* are 100% identical at the protein level and 94.8% at the nucleotide level. The genomic structure of the mouse gene (Fig. 1) is identical to that of the rat gene. The gene spans 4.8Kb and consists of six exons. The translation start site (AUG) starts at the first base of exon two, the coding region for *galanin* extends across exons three and four with the stop codon (UGA) in the middle of exon six.

25

Using the 14Kb subclone described above, a positive/negative selection targeting vector was constructed (Fig. 2). The mutation introduced removes the first five exons containing the entire coding region of the *galanin* peptide (Fig. 3). In Fig. 3:

30 A and B are the sites of the external probes used to screen the ES cells for the appropriate integration of the construct

P1 and 2 are the PCR primers used to do the same as above

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Neo = neomycin resistance gene

HSV-TK= herpes simplex virus thymidine kinase gene

5

B = BamHI

E=EcoRI

A=Asp718

Sm=SmaI

10 X=XhoI

Bg=BglII

N=NcoI

Cla=ClaI

Fsp=FspI

15

In particular, the targeting vector removes a 3.2Kb stretch of DNA and thus removes the first 5 exons of the galanin gene. The exact sites flanking the stretch of DNA removed are 5' - the Bam HI site 10bp downstream from the transcriptional start site and the 3' site is the BglII site in the middle of intron 5. These sites are indicated with asterisks in Fig. 3.

20 Other sites that could be used are the same 5' site and a differing 3' XhoI site in intron 4 which would remove only 2.9Kb of DNA and thus remove only first 4 exons.

This vector was linearised and electroporated into the E14 embryonic stem-cell (ES) line (Hooper, M. *et al* Nature. 1987; 326: 292-5). Double selection was performed over a ten  
25 day period and those clones which survived screened by PCR and subsequently by genomic mapping. In total 9 clones were identified in which one allele of the galanin gene was correctly targeted by homologous recombination and in whom karyotyping confirmed euploidy. These clones were injected into 3.5 day old blastocysts. The chimeric mice generated have been bred and germ line transmission of the disrupted galanin locus  
30 obtained from three separate ES cell clones. The heterozygous animals have been bred to homozygosity on the 129sv in-bred mouse strain and the resulting phenotype studied.

1. Results of genotype analysis of live births are in the expected ratio predicted by Mendelian genetics and the sex ratio is 1:1. Galanin levels were measured by radioimmunoassay and immunocytochemistry in areas previously demonstrated to express galanin at high levels and include brain, pituitary, spinal cord, dorsal root ganglion, stomach, small intestine and uterus. Levels in heterozygotes for the deletion were 50% of wild type controls whilst levels in the homozygotes for the deletion were undetectable in all cases.
2. Although the mutant animals grow normally after weaning compared to their wild type littermates and achieve equal adult body weights, the same is not the case if the animals are weaned two days early. At P19 (*i.e.* 19 days *post partum*) galanin would appear to be vital for the development of appetite for solids, if the animals are weaned at this point the mutants die within 48h. of starvation. Postmortem findings reveal a complete absence of food in the stomach or small bowel. Clearly this is a major finding since very little is known about the normal regulation of appetite in the peri-weaning period. The mice of the invention are useful in studies on the expression of other neuropeptides known to regulate appetite (including leptin, neuropeptide Y, CCK, CRF and GLP-1) though there are already clear differences in hypothalamic neuropeptide expression in the mutant animals at P19 compared to wild type littermates.
3. Galanin is thought to play a role in the modulation of spinal cord transmission, particularly after nerve damage (axotomy) where its expression is upregulated during axonal regeneration. The knockout mutants are clearly hypo-algesic with substantially increased response times to heat stimuli (Hargreaves model) as shown in Fig. 4. The Hargreaves Time referred to in Fig. 4 is the time taken for mouse to make a withdrawal response after an exposure to a heat lamp at a temperature of 55 ° Centigrade. The response to axotomy is attenuated in the mutants (-/-) and autotomy fails to occur whilst self-mutilation in the wild type littermates (+/+) is severe and occurs in almost all axotomised control animals (Fig. 5). The finding of hypo-algesia in the knockout mice is striking and unexpected. Previous data from Hökfelt's group in Sweden had suggested that galanin has a bimodal response on spinal cord transmission depending on the dose used.

4. Galanin has been implicated in the aetiology of Alzheimer's disease, hippocampal galanin expression is increased in cholinergic neurones as acetylcholine and choline acetyltransferase (ChAT) levels fall. Administration of galanin decreases learning behaviour in a number of mouse models, the converse is also true when galanin antagonist are infused.
- 5 Our data, thus far, has demonstrated a two fold increase in hippocampal ChAT expression in the galanin mutant mice compared to wild type controls and behavioural tests on the mice are planned.

5. Homozygote mutants enter puberty at the same time as their littermate controls, pregnancy and resulting litter size appeared unaffected. Mutant females, however, are unable to lactate and all pups died of dehydration/starvation unless fostered by wild type mothers. Pituitary prolactin content and secretion is reduced some five fold in pregnant homozygotes (-/-) compared to pregnant wild type (+/+) controls killed 4 days after birth (Fig. 6) but is only 80% of normal in randomly cycling female homozygote mice.

15

The addition of exogenous oestradiol (0.5µg of 17 β-oestradiol given subcutaneously as a suspension in linseed oil) to rodents has a strong mitogenic effect on pituitary cell number and markedly increases pituitary prolactin content (Fig.6).

- 20 These effects are abolished in the knockout mice, confirming that galanin is crucial to lactotroph growth and to prolactin secretion in the hyperoestrogenised state. These, coupled with previous data that galanin induces growth of the lactotroph, combine to substantiate the hypothesis that an activating mutation in the pituitary galanin receptor may be responsible for the formation of prolactinomas (prolactin secreting pituitary tumours).

25

It would be expected that the mutant mouse of the invention would have high insulin and low plasma glucose. Thus galanin antagonists might be of use in treatment of diabetes mellitus.

- 30 Galanin may inhibit hypothalamic somatostatin release thus stimulating growth hormone. One would expect the mutant mice to have high levels of somatostatin, low GH and to be small. Thus galanin might be a treatment for idiopathic small stature.

Such changes caused by the mutations to the mouse of the invention as disclosed above have implications for possible treatments of a number of human conditions/diseases using either galanin agonists or antagonists. Such diseases may include:- anorexia, obesity,  
5 painful neuropathies, pituitary prolactin secreting tumours, Alzheimer's dementia and diabetes.

The invention features the generation of a mutant mouse carrying a loss-of-function germ-line mutation of the galanin locus. The inactivating mutation has been introduced  
10 into the mouse genome utilising targeted mutagenesis in embryonic stem cells by homologous recombination. The mutation when bred to homozygosity on the inbred 129sv background affects feeding behaviour, lactation and pain sensitivity. The mutation may also affect memory and behaviour, sexual reproduction and fertility and insulin secretion with resultant changes in circulating blood glucose levels.

## CLAIMS

Final version

## CLAIMS

1. A mammal which lacks a functional galanin gene.
- 5 2. A mammal according to claim 1 in which the galanin gene has been inactivated.
3. A mammal according to claim 1 or 2 in which the galanin gene has been inactivated by at least partial deletion.
- 10 4. A mammal according to claim 3 in which the portion of the galanin gene between the Bam HI and Bgl2 restriction sites asterisked in Fig. 3 has been deleted.
5. A mammal according to claims 1, 2, 3 or 4 which is a rodent.
- 15 6. A rodent according to claim 5 which is a mouse.
7. Tissue, cells and cell lines derived from a mammal, rodent or mouse according to any preceding claim.
- 20 8. Tissue, cells or cell lines according to claim 7 which are cells from pancreas, pituitary, cortex, dorsal root ganglia or are derived from such cells.
9. The use of a mammal, rodent or mouse according to any one claims 1 to 6 or tissue, cells  
25 and cell lines according to claim 7 or 8 in an assay to determine a biological effect of galanin.
10. The use according to claim 9 in which the biological effect is selected from diabetes and insulin secretion, appetite, growth hormone effects lactation, prolactin oversecretion,  
30 pain sensitivity, memory, behaviour, sexual reproduction and fertility.
11. An analgesic composition comprising a galanin antagonist.

12. The use of a galanin antagonist in the preparation of a medicament for the treatment of pain.
13. A method of suppressing pain in a mammal, the method comprising administering a galanin antagonist to that mammal.
14. The use of a galanin antagonist in the preparation of a medicament for the treatment of painful neuropathy.
15. An appetite suppressant composition comprising a galanin antagonist.
16. The use of a galanin antagonist in the preparation of a medicament for the suppression of appetite.
17. A method of suppressing appetite in a mammal, the method comprising administering a galanin antagonist to that mammal.
18. An anaesthetic composition comprising a galanin antagonist.
19. The use of a galanin antagonist in the preparation of an anaesthetic composition.
20. A method of anaesthetising a mammal, the method comprising administering a galanin antagonist to that mammal.
21. A lactation suppression composition comprising a galanin antagonist.
22. The use of a galanin antagonist in the preparation of a medicament for the suppression of lactation.
23. A method of suppressing lactation in a mammal, the method comprising administering a galanin antagonist to that mammal.

## CLAIMS

Final version

24. A composition comprising a galanin antagonist for the treatment of prolactinoma in a mammal.

25. The use of a galanin antagonist in the preparation of a medicament for the treatment of prolactinoma.

26. A method of treating prolactinoma in a mammal suffering from prolactinoma, the method comprising administering a galanin antagonist to that mammal.



## GALANIN

This invention relates to galanin and its uses. In particular, the invention provides a knockout mouse which lacks a functional galanin gene. The mouse may be used to investigate the  
5 effects of galanin.

It has also been unexpectedly discovered that galanin antagonists may be used in the management of pain, particularly painful neuropathy, suppression of pain, suppression of lactation, and treatment of prolactinoma.



# GENOMIC STRUCTURE *mGAL*

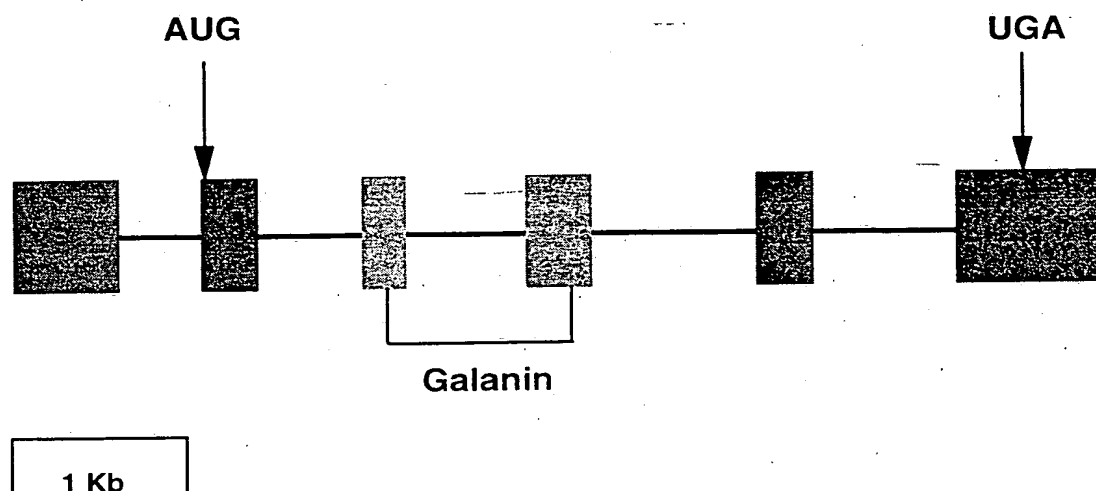


Fig. 1



## TARGETING VECTOR

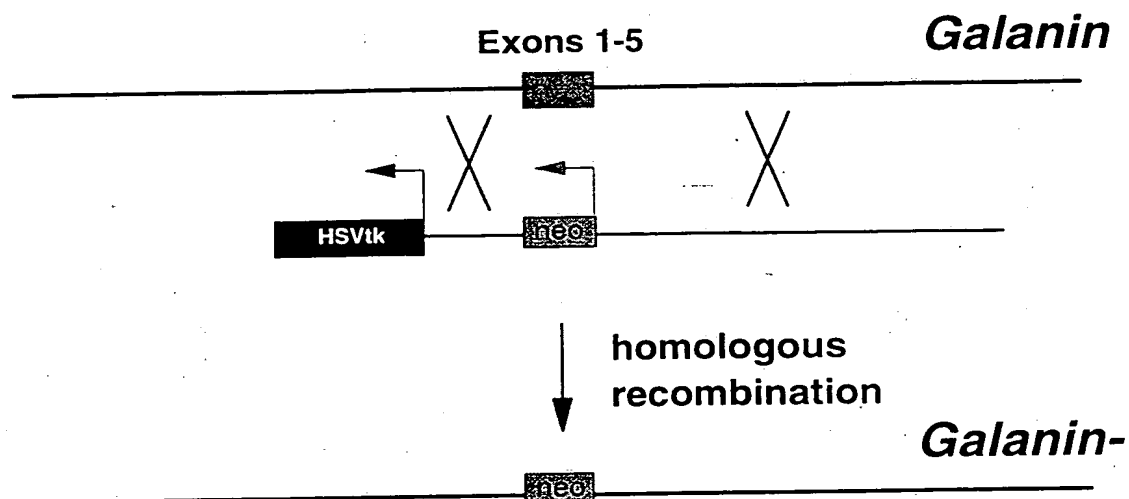


Fig. 2



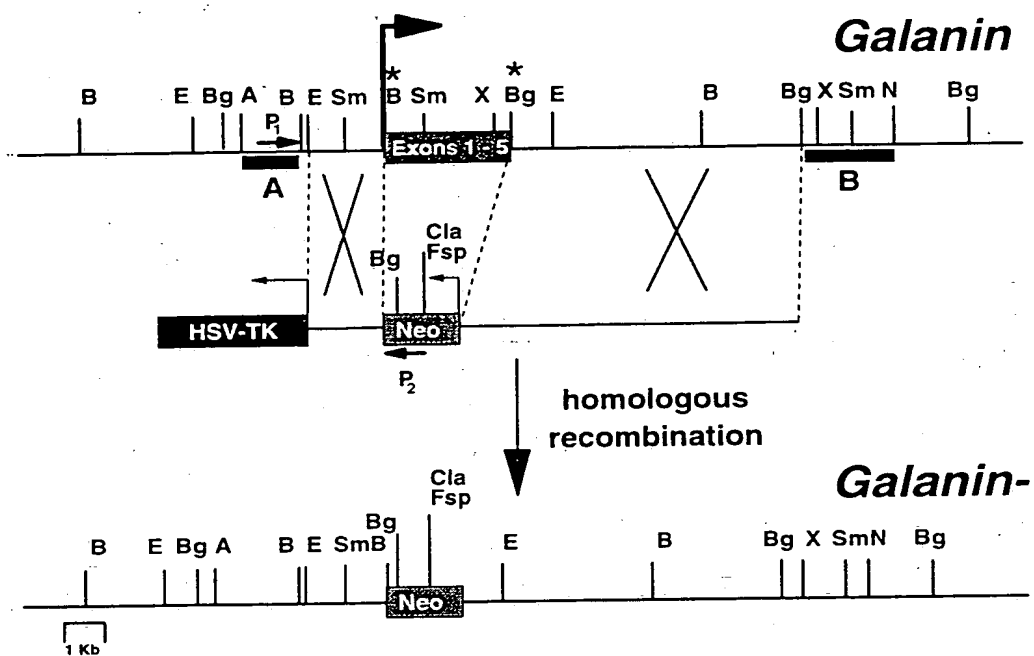


Fig. 3





## Effect of Galanin Disruption on Heat Sensitivity

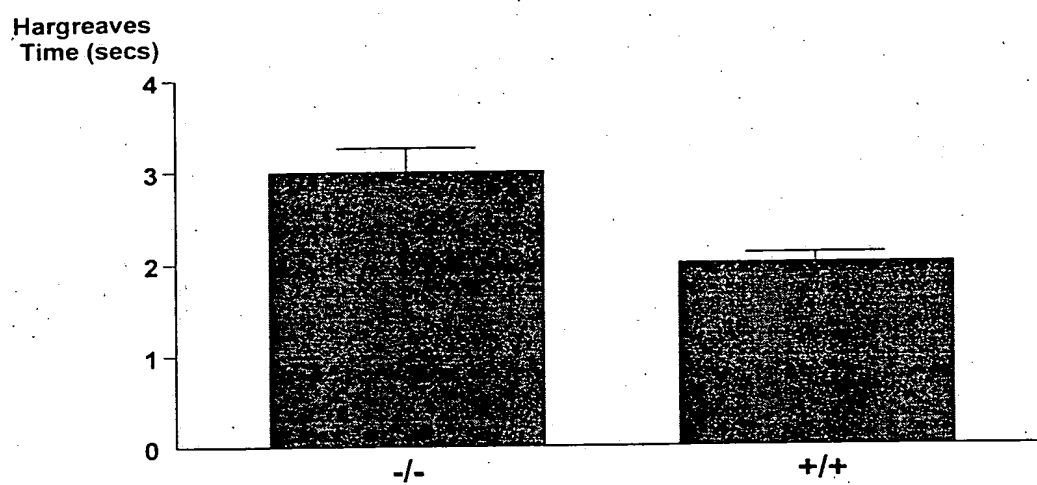
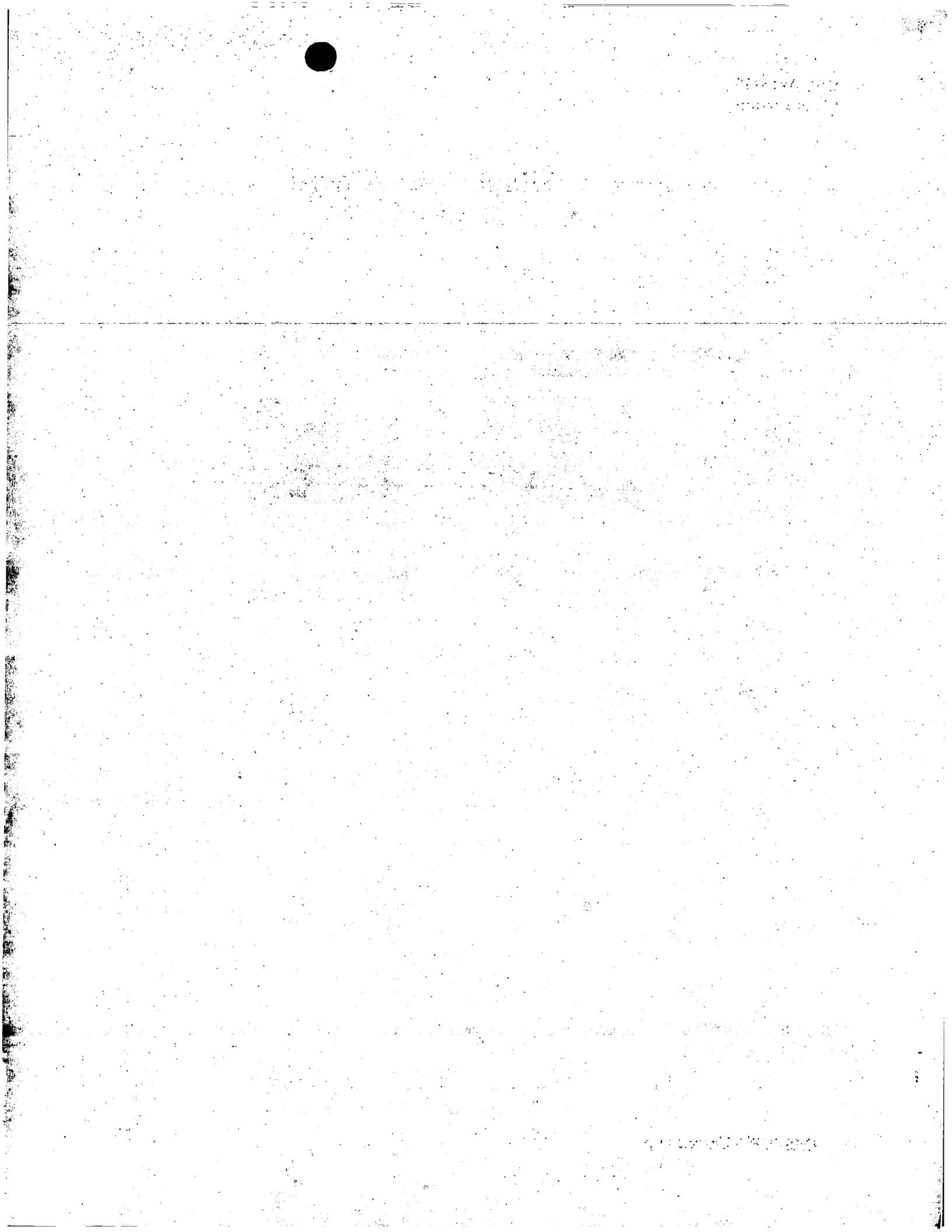


Fig. 4



1974-1975  
1976-1977



## Autotomy Behaviour After Sciatic Nerve Section

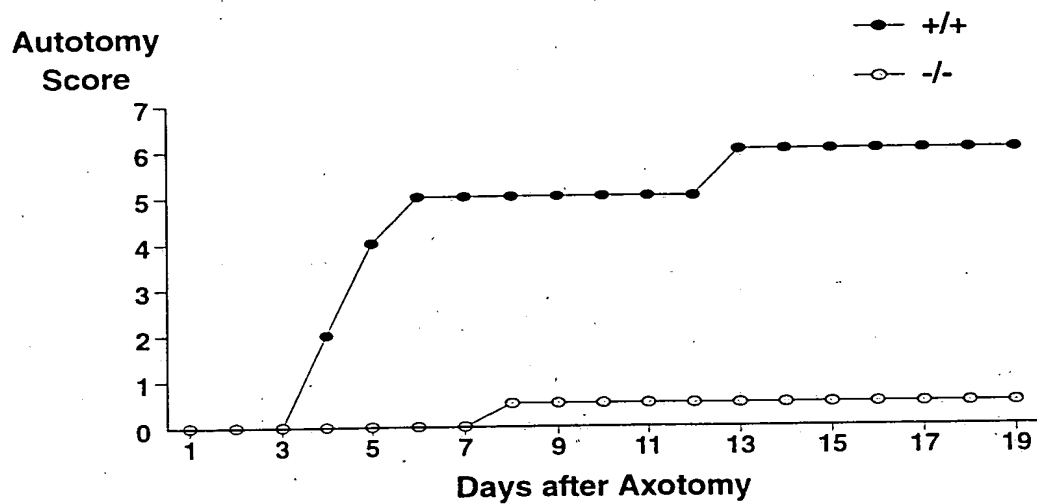
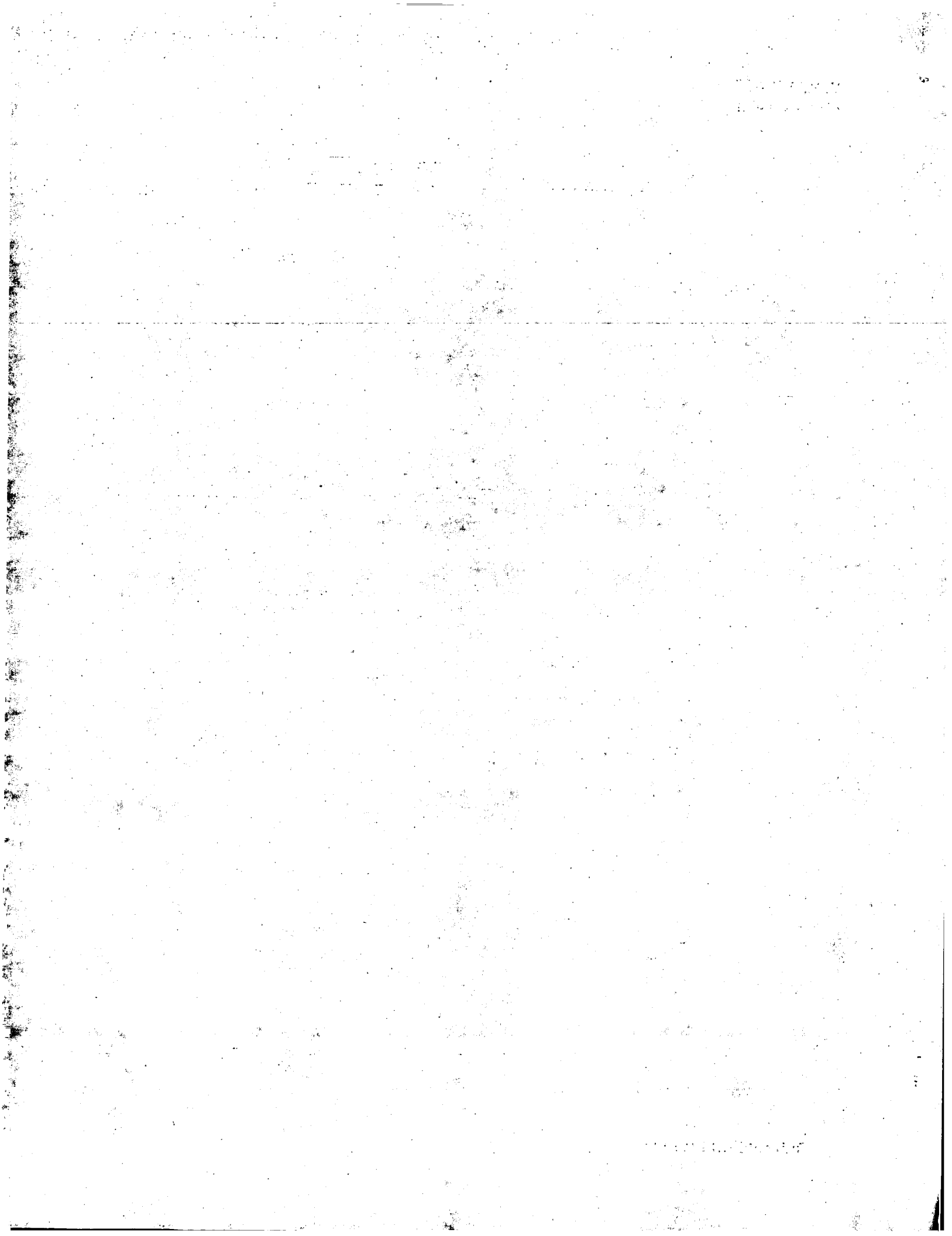


Fig. 5



## ANTERIOR PITUITARY CONTENT

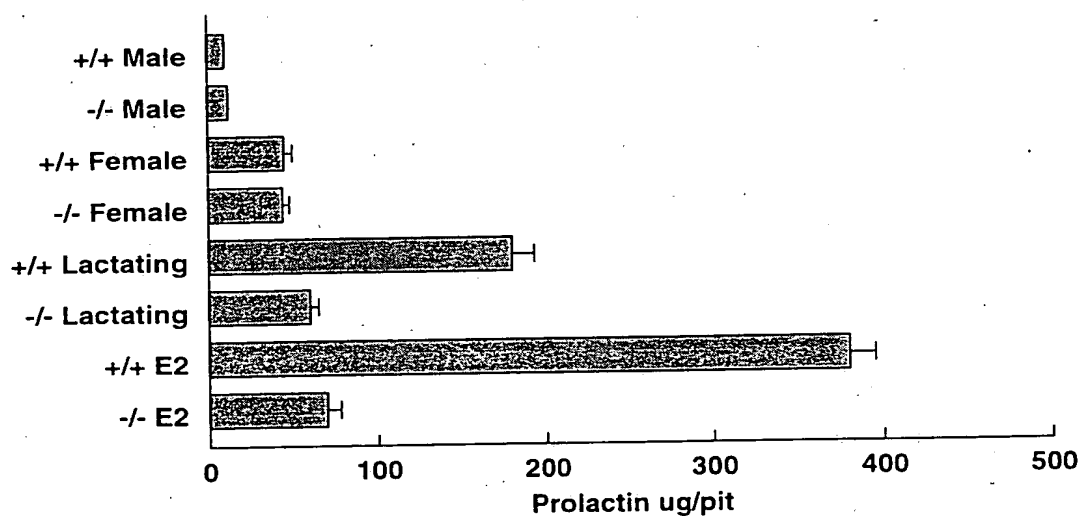


Fig. 6



## ANTERIOR PITUITARY CONTENT

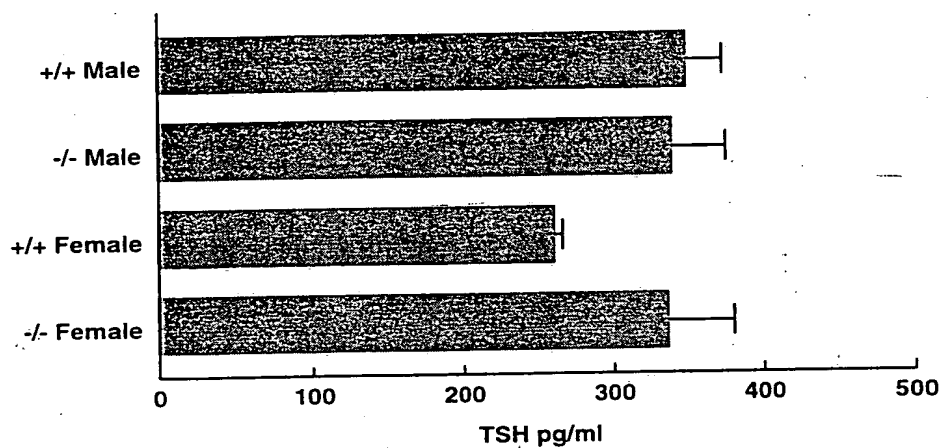


Fig. 7





## ANTERIOR PITUITARY CONTENT

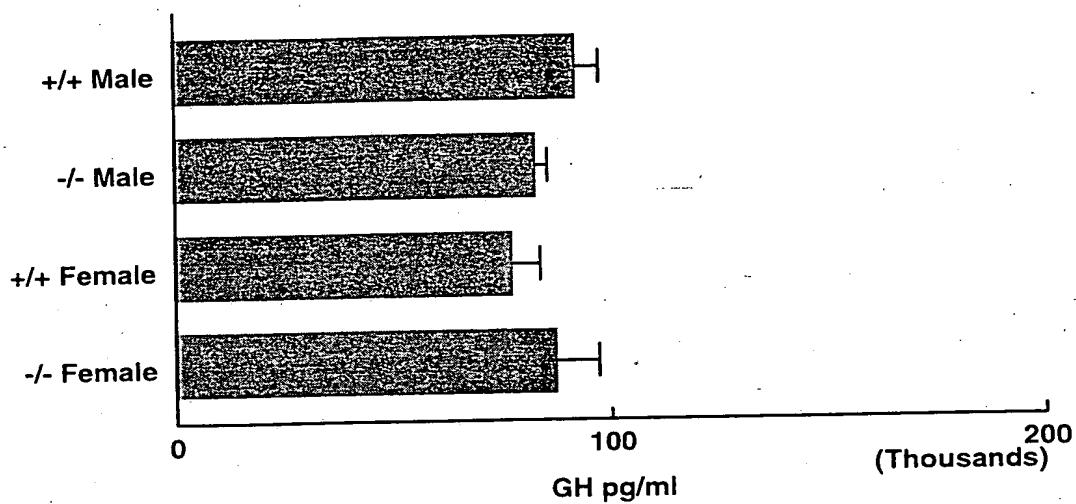


Fig. 8



## ANTERIOR PITUITARY CONTENT

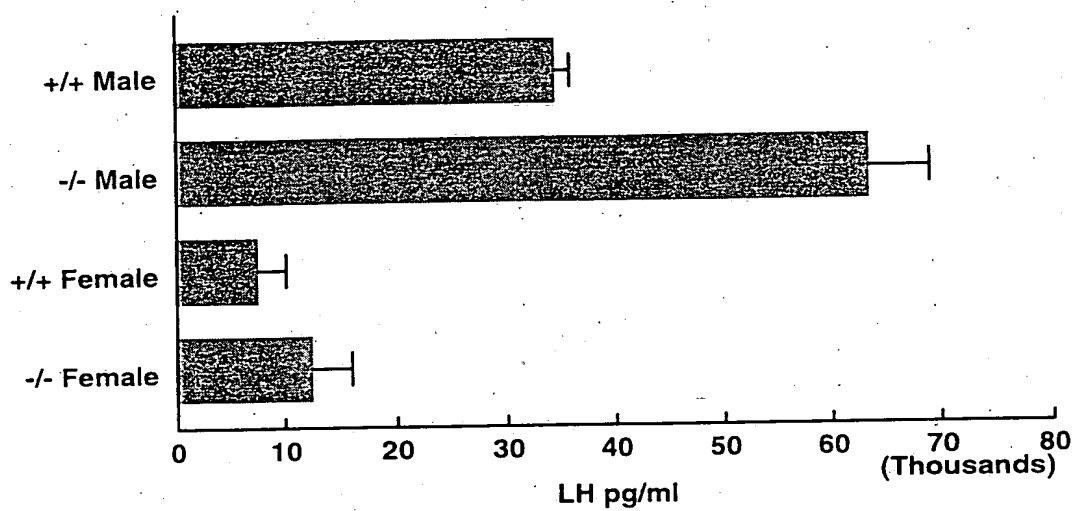


Fig. 9

PCT/9397/01991

WITNESS & FORGERS

24 July 1997